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Developing bacteriocins of lactic acid bacteria into next generation biopreservatives

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Running Title: Developing LAB bacteriocins

Abstract

Bacteriocins are ribosomally synthesized peptides produced by bacteria which can kill other bacteria. Those produced by lactic acid bacteria (LAB) are of great interest as they are often employed in food processing and food fermentations as natural biopreservatives. In this review, we discuss the implementation of bioengineering to enhance the antimicrobial activity, antibacterial spectrum and physico-chemical properties of LAB bacteriocins. Additionally, we discuss the potential applications of bacteriocin derivatives for use as promising food preservatives alone or in combination with other naturally derived antimicrobials as a form of hurdle technology and the regulatory status of strains engineered through food-grade approaches.

Highlights

- Bioengineering can generate novel bacteriocin variants for specific purposes.
- Genome mining has identified new bacteriocin biosynthetic gene clusters.
- Bioengineered bateriocins show great promise as synergists in hurdle technology.
- Bacteriocin producing strains which have been tailored through food-grade methods can be directly added to food.

Introduction

The growth in world population and the globalization of food commerce has led to large scale food production practices requiring ever longer transport networks and extended storage times until final distribution to consumers. In addition, growing consumer demand for food products that are minimally processed and free from chemical additives presents a complicated and difficult challenge for food processors. Such demand has opened up new opportunities for the use of natural antimicrobials derived from plant, animal or microbial sources to control the growth of undesirable micro-organisms in food [1,2]. Bacteriocins (ribosomally-produced, small, heat-stable peptides that are active against other bacteria) provide one potential solution. While bacteriocins can be produced by a range of Gram-positive and Gram-negative bacteria [3], those produced by lactic acid bacteria (LAB) are of particular interest to the food industry for several reasons. Firstly, members of the LAB group have a history of safe use as starter cultures in food fermentations and many possess "Generally Regarded as Safe (GRAS)" status according to the US Food and Drug

Administration [4]. Secondly, besides being non-toxic to eukaryotic cells, LAB bacteriocins are extremely potent against many food spoilage microbes and pathogenic bacteria, demonstrating killing activity in the nanomolar range. Thirdly, they do not interfere with the sensory quality of foods. Finally, the ribosomal origin of bacteriocins has enabled the manipulation of the associated structural gene in a more direct fashion than is possible for other classes of antimicrobials to obtain variants with potentially beneficial properties. Indeed, several groups have reported on the enhancement of bacteriocins' performance in food environments, including the engineering of derivatives with enhanced activity and inhibition spectra, increased resistance to proteolytic enzymes as well as the combination of such derivatives with other natural antimicrobials in the form of hurdle technology. This review will focus on recent developments with regard to these achievements and present the latest innovations which aim to harness the full potential of these highly potent antimicrobials.

Classification of LAB Bacteriocins

Bacteriocins produced by LAB represent a heterogeneous group of peptides encoded by a diverse genetic repertoire. Some are post-translationally modified and this aspect, together with their mode of action, has traditionally been used as a basis for their classification [5]. The simplest scheme comprises two classes; i.e. Class I bacteriocins also known as RiPPs (Ribosomally Produced and Post-translationally modified Peptides) encompasses all the peptides that undergo enzymatic modification during their biosynthesis (including lanthionines, glycosylation and/or heterocycles). Class II do not contain unusual modifications. However, recent extensive genome mining analysis of LAB suggests that the repertoire of antimicrobials that are encoded in publicly available sequence databases could be even more extensive than previously thought, with some putative classes thus far not reported in LAB (e.g. lasso peptides and sactipeptides) [6]. Furthermore, an *in silico*

screening approach of genome-sequenced isolates from the human gastrointestinal tract (GIT) identified more than 70 clusters of note from almost 60 unique members including Firmicutes, Bacteroidetes, Actinobacteria, and others [7]. The most commonly identified class of bacteriocin was the >10 kDa class, formerly known as bacteriolysins, followed by lantibiotics and sactipeptides [7]. Consequently, a revised scheme (that is also valid for bacteriocins from non-LAB micro-organisms) proposes three classes in which Class I is divided into six subclasses representing different modifications, Class II comprises four subclasses of unmodified peptides of <10kDa and Class III are large-molecular-weight (>10kDa) proteins and are subdivided into the bacteriolysins and the non-lytic bacteriocins [6].

Bioengineering to modulate the physicochemical properties of LAB bacteriocins

Despite the amount of research that has been carried out on the discovery, characterization and mode of action of LAB bacteriocins over the last few decades, to date, just a handful have been commercialized to any extent. These include nisin, a lantibiotic peptide produced by *Lactococcus lactis* [8], pediocin PA-1 produced by *Pediococcus acidilactici* [9] and carnocyclin A produced by *Carnobacterium maltaromaticum* UAL307 [10]. However, studies with other LAB bacteriocins including enterocin AS-48 [11] or lacticin 3147 [12] demonstrate their enormous potential as biopreservatives in food. Nisin is used in most major food-producer countries as a concentrated fermentate powder (e.g. Nisaplin) in a wide variety of dairy and non-dairy products to control the growth of Gram positive bacteria [13]. A fermentate powder produced from the pediocin-producing strain *Pediococcus acidilactici* (ALTA 2351, Kerry Biosciences, Ireland) can be used to protect meat products from *L. monocytogenes* contamination [14]. Carnocyclin A is marketed as Micocin in the US and Canada and has been developed to inhibit *Listeria monocytogenes* in ready-to-eat meat (RTE) products [10]. Accordingly, these bacteriocins have been the subject of several

bioengineering strategies (For comprehensive reviews see [15-17]) that have sought to improve bacteriocin efficacy in the food environment. Peptide function can be influenced by a number of factors including fat content, proteolytic degradation, polar or non-polar food components, pH (which influences the solubility of the bacteriocin) and sodium chloride concentrations [18]. For example, the limited activity spectrum of nisin with respect to pH and its intrinsic insolubility has emphasized the need for alternative versions that exhibit superior stability and are suitable for food fermentation and preservation practices. The natural variant nisin Z, which differs from nisin A by one amino acid (asparagine rather than histidine at position 27) provides an example of a derivative with improved functional characteristics since, while it has similar antimicrobial activity to nisin A, nisin Z displays a higher rate of diffusion [19] and is less soluble at low pH [20]. Recently, bioengineered nisin derivatives were identified with an enhanced ability to diffuse through complex polymers that enabled the peptides to surpass nisin A in restricting growth of *Listeria monocytogenes* in commercially produced chocolate milk containing carrageenan as a stabilizer [21]. Despite the fact that derivatives of the unmodified class II bacteriocins can be generated with relative ease [22], there are comparatively few examples of instances in which LAB producers of the class II bacteriocins have been engineered to positive effect. However, in the case of pediocin PA-1/AcH, greater resistance to oxidation was achieved by the replacement of a methionine moiety with a hydrophobic one, which had only minor effects on antimicrobial activity [23]. Such modifications are an important step in the development of pediocin PA-1 into an advantageous food additive. Some studies have aimed to incorporate protease resistance into LAB-derived peptides. For example, particular modification of trypsin recognition sites in the class IIb bacteriocin salivaricin P produced by *Lactobacillus salivarius* had only minor effects on activity [24].

Bioengineering to modulate the antimicrobial activity and spectrum of LAB bacteriocins

The range of inhibitory activity by LAB bacteriocins can be either narrow, inhibiting only those strains that are closely related to the producer organism, or wide, inhibiting a broad range of Gram-positive micro-organisms [25]. Several investigations have sought to identify nisin derivatives that are enhanced with respect to the purpose for which nisin is most renowned, the inhibition of Gram-positive bacteria [15]. One remarkable derivative, nisin A M21V (Nisin V) (Fig. 1) exhibits enhanced potency against a wide range of targets, most notably *L. monocytogenes.* Furthermore, this enhanced activity was apparent in food model experiments with purified peptide [26]. *L. monocytogenes* is of major concern to the food industry. Apart from the risk to human health, food product recalls due to *Listeria* contamination present an enormous financial burden, estimated to be in the billions of dollars per year in the United States [27]. Recently, nisin V in the form of a fermentate, combined more effectively than nisin A with the essential oils carvacrol, thymol and trans-cinnamaldehyde to inhibit *L. monocytogenes* in a validated food model system [28]. Worryingly, *L. monocytogenes* has the ability to form biofilms providing it with the means to survive on contact surfaces, with higher levels of resistance to disinfectants and potential for growth under the rigorous conditions used for food processing. This could lead to contamination of food products. A recent study demonstrated the effectiveness of nisin M21A, in combination with natural food-grade additives, in targeting biofilms of *L. monocytogenes* F6854 [29], a strain that has been associated with contaminated turkey frankfurters. Similarly, the advantage of shortening or extending the hinge region of nisin has generated variants with improved bioactivity against one or more indicator targets, including *L. monocytogenes, E. faecalis and B. sporothermodurans* [30]. Bioengineering to enhance the efficacy of Class IIa bacteriocins has also been considered. Mutated peptides in which

residues were substituted within the N-terminal half of pediocin PA-1 exhibited increased activity against *Micrococcus luteus* and *Staphylococcus aureus* [31], while variants within the C-terminus displayed increased potency towards *L. monocytogenes* [32]. Directed mutagenesis studies have been employed to manipulate enterocin AS-48, a broad-spectrum circular bacteriocin produced by a strain of *E. faecalis* [33]. The authors determined that enterocin AS-48 was active in a dimeric form, and established the essential residues involved in these interactions [34]. Such knowledge may facilitate the design and expression of novel variants with improved antimicrobial activity. Notably, the successful chemical synthesis of circular bacteriocins including enterocin AS-48 has recently been achieved [35], providing a more suitable means to generate novel variants, the production of which can often be compromised in the native producer.

The poor activity of LAB bacteriocins toward Gram negative bacteria is due to the outer membrane (OM) of the Gram negative cell wall [36]. The OM functions as an efficient permeability barrier and is able to exclude macromolecules such as bacteriocins or enzymes. Importantly, bioengineered nisin variants S29A and S29G have been shown to display improved activity against Gram negative bacteria [37]. Moreover, while nisin A has been shown to be effective against Gram negatives when used in combination with chelating agents such as EDTA [38], perhaps a more attractive option for food applications is the combination of nisin with natural phytochemical compounds such as essential oils, which act by permeabilization/disruption of the OM. [39]. Recent investigations have also sought to extend the antimicrobial spectrum of pediocin-like bacteriocins to include Gram-negative bacteria. The enterocin CRL35, a pediocin-like bacteriocin, has a potent antilisterial activity but is inactive against Gram-negative targets. In contrast, microcin V (previously known as colicin V) is specifically active against Gram-negative bacteria [40]. A hybrid bacteriocin named Ent35–MccV resulting from the gene fusion of the enterocin CRL35 and microcin V

genes (*munA* and *cvaC*, respectively) displayed inhibitory activity against *E. coli*, *L. monocytogenes*, and other pathogenic Gram-positive and Gram-negative bacteria [41]. Synthetic biology approaches are another promising means to provide insights into structure-stability relationships and generate novel derivatives with improved function. For example, analogues of Lacticin 481 containing non-proteinogenic amino acids were found to have enhanced antibacterial activity [42].

Heterologous expression to increase yield and multiple bacteriocin production

One of the issues that remains to be tackled is that of bacteriocin production, which can be low or inconsistent in lactic acid bacteria and which can be affected by poor growth of producing strains in particular food environments. Although the heterologous production of bacteriocins by LAB is reliant on several factors, bioengineering techniques can facilitate increased levels of bacteriocin production. Some efforts to increase bacteriocin yield have involved using synthetic genes encoding bacteriocins cloned and expressed in yeasts. For instance, the use of codon optimization was recently employed to overcome the bottleneck of low yield of Enterocin A, a class IIa bacteriocin produced by *Enterococcus faecium* CTC492 [43]. Likewise, a recent study involving synthetic biology approaches describes the development of a genetic system that facilitates significant overproduction of nisin [44]. Such innovative systems could potentially reduce the cost of bacteriocin production and also

provides a means by which sufficient quantities of bacteriocin can be produced *in situ*. In addition, novel bacteriocin clusters identified through genome mining and considered highly advantageous to the food industry could be cloned and expressed in suitable hosts. In fact, bacteriocin producing bioprotective cultures that target particular pathogens are commercially available under various trade names [45]. Remarkably, naturally occurring multi-bacteriocin producing LAB have been reported [46]. Consequently, the use of bacteriocin combinations or bacteriocin 'loading' may represent a useful approach whereby an assortment of bacteriocins produced *in situ* provide an effective cocktail that can act synergistically to inhibit desired target pathogens. Indeed, some optimistic reports are emerging on the use of multi-bacteriocin mixtures for the effective control of foodborne pathogens such as *Listeria* [47,48].

Bioengineering and the Regulation of Genetically Modified Micro-organisms

Although the application of bioengineering has been instrumental in the fundamental analyses of bacteriocin biology, mode of action studies, and the ability to design more potent peptides with enhanced properties and target selectivity, the application of such peptides as food preservatives may face a significant regulatory obstacle in some jurisdictions. The genetic manipulation of bacteriocins or the producer strains needs to pass through strict safety regulations and guidelines laid down by regulatory agencies such as the FDA (or the European Food Safety Authority [EFSA] in Europe) for approval to be used in human consumption. Indeed, some of the strategies employed to bioengineer many of the bacteriocins described above involve methods that could result in the producer being labelled as a genetically modified micro-organism (GMM). Alternatively, self-cloning of non-pathogenic micro-organisms is not considered to lead to a GMM so long as containment of

the organism is guaranteed (directive 90/219/EC). Notably, the temporary introduction of plasmid vectors or the use of recombinant vectors with an extended history of safe use in the particular micro-organisms, or introduction of DNA from another micro-organism belonging to the same species fall within the definition of self-cloning. Thus, minimal changes to bacteriocin structural genes (such as the alteration of single codons) made using food grade strategies [26,49] fall outside the remit of the EFSA Contained Use legislation and therefore are not regulated as GMMs.

Conclusions

A broad range of technologies have emerged in recent years that provide a battery of valuable tools to expand the potential of bacteriocinogenic strains for food applications. The knowledge gained will improve our understanding on the global effects of bacteriocins in food ecosystems and permit more rational approaches for their application in foods. Several bioengineered bacteriocins capable of inhibiting food-associated Gram positive and Gram-negative bacteria of concern have been recently described. A number have already been tested with satisfactory results in terms of the control of pathogenic bacteria in model food systems. Moreover, their use in combination with other naturally derived antimicrobials in the form of hurdle technology may open new possibilities for the control of a broad range of undesirable organisms. Although genetic manipulation by recombinant and bioengineering-based approaches hold great promise, only bacteriocins which have been tailored through food-grade methodologies can be directly added to food. Furthermore, as the number of microbial genome sequences has increased exponentially, an even larger collection of

putative bacteriocin biosynthetic gene clusters has been revealed. These clusters can be used to identify producer strains, or the information gained from their analysis can be used indirectly to guide the bioengineering of new and existing peptide structures with enhanced functionality for use in the food industry.

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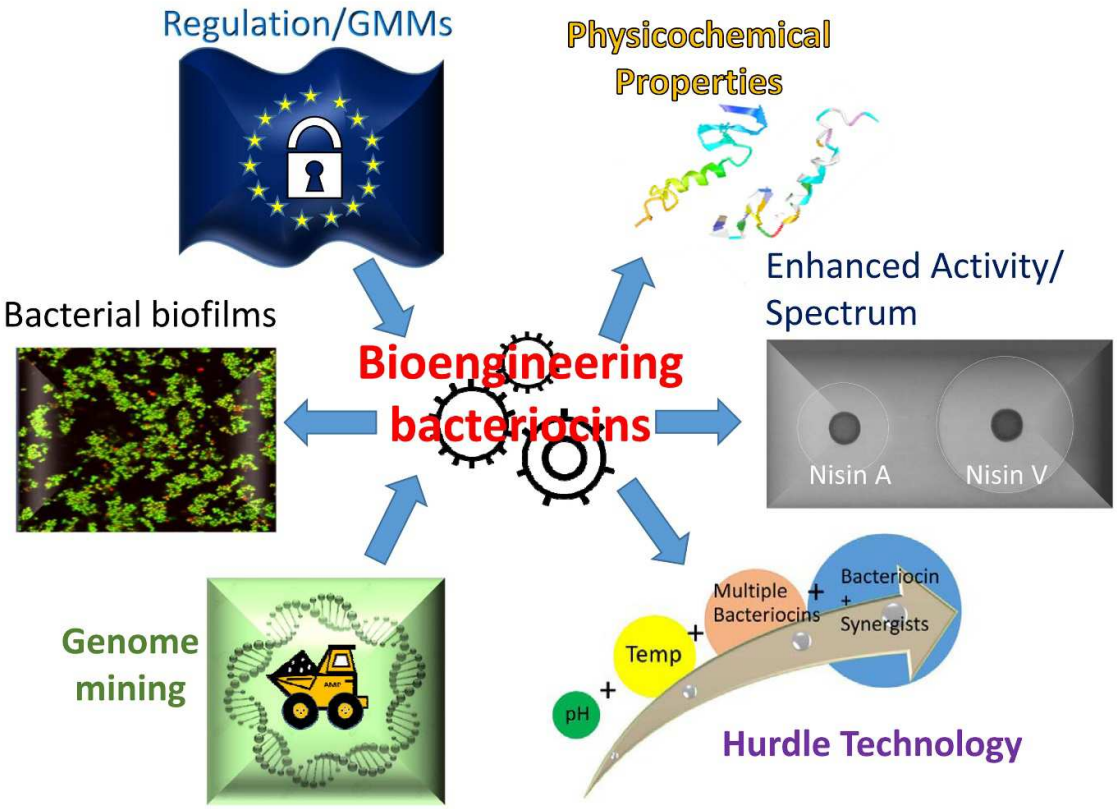

Figure 1. Bioengineering bacteriocins: bacteriocins with improved physicochemical properties (pH, solubility) have been generated as well as novel variants with improved activity, target spectrum and anti-biofilm efficacy. Bioengineered variants can act synergistically to inhibit desired target pathogens in the form of hurdle technology. Genome mining has identified an even larger collection of new bacteriocin biosynthetic gene clusters which can be used to guide the bioengineering of new and existing peptide structures. Bioengineered strains which have been tailored through food-grade approaches are not considered GMMs and can be directly added to food.